ANTIVESICANT STRATEGIES BASED ON DNA REPAIR AND APOPTOSIS

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ABSTRACT

DNA is a major cellular target of the vesicant chemical warfare agent sulfur mustard (SM, bis-(2-chloroethyl) sulfide). Others and we have proposed a possible role of apoptosis in SM vesication. Our results suggest that in SM-exposed human epidermal keratinocytes (HEK), DNA damage, DNA repair, and apoptosis may be interdependent. In HEK, SM causes cell death accompanied by caspase-3 activation indicating apoptosis. The general caspase inhibitor Z-VAD-FMK (benzyl oxycarbonyl-Val-Ala-Asp (o-methyl-fluromethylketone)) decreases not only SM-induced apoptosis, but also protease stimulation and consequent degradation of laminin-5 which maintains epidermal-dermal junction integrity. This knowledge may, therefore, be useful in developing successful antivesicant strategies.

INTRODUCTION

In present day military conflict, the use of chemical warfare agents is a grave concern. The major chemical agents are nerve agents, soman, sarin, tabun and VX; blister agents, sulfur mustard and lewisite; blood agents, hydrogen cyanide and cyanogen chloride; and respiratory agents, phosgene and diphosgene. Here we discuss sulfur mustard (SM), which has been used in previous wars and is an identified future threat for not only military application but also civilian terrorism. SM is highly reactive and extremely toxic. Depending on the route (liquid or vapor or both) and dose of exposure, SM may damage exposed tissues such as skin, eyes and lungs as well as internal tissues such as blood cells and the nervous system. Documented SM effects are skin blisters, eye lesion, lung inflammation, depression of blood cell counts, particularly white blood cells, and to some extent abnormal mental functions, and others. This account indicates the complexity and the diversity of SM action, which encompasses effects on organs that are either exposed or unexposed to the environment. One common cause of pathology due to SM, i.e., its

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vesicating effect that applies to the exposed organs, namely, the skin, eyes and lungs, is the damage to the epithelium of the respective organs. This epithelial damage is characterized by cytotoxic events leading to cell death in the basal cell layer, followed by a separation of the epithelium from the substratum. To develop effective protection against SM, researchers have studied its different possible mechanisms of action contributing to cytotoxicity and cell death. The results obtained indicate that DNA damage and its consequences including cell death are most relevant to the vesicant action of SM. Our studies were focused on the blistering mechanism of SM in the skin and, therefore, were conducted in either the cultured human epidermal keratinocytes (HEK) model or in an experimental animal skin model. At present, due to our lack of understanding of the exact mechanisms by which SM causes skin blisters there is no effective prophylactic or therapeutic intervention. Our studies and analyses were based on the hypothesis that among the different cell death mechanisms, apoptosis or programmed cell death is the initial sequel of the toxic mechanisms due to SM contributing to its vesicant action. In SM toxicity, DNA damage and its repair is an important determinant of apoptosis. Here, we present arguments based on theoretical considerations as well as experimental results in favor of considering an antiapoptotic antivesicant approach.

RESULTS AND DISCUSSION

Kan *et al*. (2003) studied cell death and microvesication *in vivo* in hairless guinea pig skin areas exposed to SM vapor for 8 minutes. By immunohistochemical detection of basal cell apoptosis using the ApopTag in situ labeling, they observed a time-dependent increase in apoptotic cells in exposed sites. Apoptotic basal cells were very few or absent at unexposed sites or at exposed sites after 3 hours. However, at exposed sites, apoptotic basal cells increased with time as observed at 6 hours (18%) and 12 hours (59%). After 24 hours, necrotic cells were so numerous that apoptotic cells could not be easily distinguished. In these experiments, it was noteworthy that following SM exposure, apoptotic basal epidermal cells appeared as early as 6 hours when the epidermal-dermal junction was intact and there was no visible microvesication. However, between 6 hours and 12 hours, when the apoptotic cell population increased from 18% to 59%, there were clearly visible microvesicles lined with apoptotic basal cells in the epidermal section. These results strongly suggest that in SM-exposed skin, epidermal basal cell apoptosis may be an early and critical event responsible for microvesication. An apparently valid mechanism of SM vesication is that SM-exposed basal epidermal cells begin to die via apoptosis and detach from the dermis due to the degradation of the epidermal-dermal attachment components, resulting in the separation of the epidermis from the dermis and, therefore, vesication. Below we present our observations in support of this proposed biochemical mechanism of SM-induced apoptosis and its relation to DNA damage and repair in HEK.

Cell death may occur via two discernible mechanisms, oncosis and apoptosis. The morphological pathways of these two processes are as follows. In oncosis, cells swell, progress to cytoplasmic blebbing, and finally to necrosis. On the contrary, apoptotic cell death is characterized by cellular shrinkage along with nuclear chromatin condensation, and then nuclear fragmentation resulting in the formation of apoptotic bodies containing membrane-bound chromatin fragments. A physiologically significant difference between oncosis and apoptosis is that oncosis triggers inflammation, but apoptosis does not. Moreover, it should be noted that

both oncosis and apoptosis terminate in a common status, i.e., necrosis. However, oncotic necrosis and apoptotic necrosis display differences in morphological features. As discussed above, at least in the hairless guinea pig skin model, SM toxicity displays an early onset of epidermal basal cell apoptosis that progresses with time to a status of predominantly necrosis via an apoptotic-necrotic continuum. We may, therefore, reason that since the SM-induced cell death process appears to begin with apoptosis, an antiapoptotic intervention may be a rational antivesicant approach. As will be shown later, the validity of this concept has been demonstrated in an experimental animal model (Rosenthal *et al*., 2003).

Previously, we described the diversity and the complexity of the possible biochemical mechanisms of SM toxicity (Ray *et al*., 2002). We proposed that these mechanisms could involve (a) disturbed intracellular Ca^{2+} homeostasis, (b) protease stimulation, (c) phospholipase stimulation and consequent release of arachidonic acid that could be further metabolized to inflammatory mediators, (d) DNA damage-induced poly (ADP-ribose) polymerase (PARP) activation and associated events such as oxidative stress, protease stimulation, lipid peroxidation etc., and (e) DNA damage-induced apoptosis via increase in PARP, p53 and Fas (death receptor) as well as Fas ligand. Here, we focus particularly on the DNA damage and repair-dependent mechanisms related to apoptosis.

In SM-exposed HEK, DNA damage increases PARP, FasL, and plasma membrane associated Fas receptors (Rosenthal *et al*., 1998). Elevated PARP is believed to initiate an intrinsic mitochondrial mechanism of apoptosis involving Ca^{2+}-CaM, cytochrome C and caspases. Binding of FasL to Fas receptors initiates an extrinsic apoptotic mechanism via a cascade of molecular events involving an adaptor protein containing FADD (Fas associate death domain) and caspases. DNA damage also activates DNA repair mechanisms involving among other factors, DNA ligase, DNA-dependent protein kinase (DNA-PK) and the transcription factor p53. DNA-PK is known to activate p53 via phosphorylation. We observed that activated p53 is elevated in SM-exposed HEK (Rosenthal *et al*., 1998), and the p53 inhibitor curcumin inhibits SM-induced apoptosis in HEK (Ray *et al*., 2001). As will be explained later, we also have experimental evidence to support that in SM-exposed HEK, DNA damage/repair and apoptosis are interdependent.

SM may cause apoptosis via several pathways dependent on different cellular effects such as (a) intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase, (b) Ca^{2+}-CaM activation (mitochondrial), (c) energy depletion, and (d) Fas/FasL elevation (Ray *et al*., 2002). Among these, the Ca^{2+}-CaM mediated mitochondrial pathway and the Fas/FasL mediated pathway have been experimentally validated (Rosenthal *et al*., 1998; Rosenthal *et al*., 2003). These different pathways act by activating respective pathway-specific caspases. However, all of these pathway-specific caspases ultimately activate the executioner caspase-3 that causes PARP cleavage and nuclear breakdown, and finally apoptosis. A rational antiapoptic approach may, therefore, be either (a) to prevent the initial signal(s), e.g., Ca^{2+}-CaM and/or Fas/FasL elevation, or (b) to inhibit the pathway-specific caspases and/or the executioner caspase-3. Our results suggest that both approaches are possible and useful. Our results also suggest that besides these mechanisms that directly regulate apoptosis other related mechanisms, e.g., of DNA damage/repair, cell cycle regulation, etc., may also be exploited to inhibit or to prevent apoptosis as discussed below.

We previously reported that SM-damaged DNA is repaired in cultured HEK (Bhat *et al*., 1997, 1999). DNA repair in SM-exposed HEK involves activation of PARP and DNA ligase I (Bhat *et al*., 2000); and DNA ligase I activation is via DNA-PK-mediated phosphorylation (Bhat *et al*., 1999). Now, we have additional evidence to propose that (a) in SM-exposed cells, DNA repair and apoptosis are interdependent, and (b) it may be possible to regulate these two SM effects by manipulating the mechanisms that link them. In replicating epidermal basal cells, SM-induced chromosomal damage, if not repaired, may cause cell death during the mitotic phase of the cell cycle. We hypothesize that cell cycle inhibition, e.g., by using a chemical inhibitor, may allow the cell to repair DNA damage and thus prevent cell death. We also hypothesize that inhibiting DNA repair should accelerate apoptosis and inhibiting apoptosis should facilitate DNA repair or result in decreased DNA damage. The following are some observations in support of these hypotheses.

Mimosine (300 μM), a compound that causes G1 block, protects against SM (500 μM)-induced cell viability loss in HeLa cells as measured by flow cytometry using propidium iodide (PI). Cells were incubated with mimosine for 2 hours prior to SM exposure, then incubated with mimosine for 1, 3, 5 or 21 hours before medium was replaced with fresh medium without mimosine. At 24 hours after SM exposure, the cells were harvested and viabilities determined using PI as the vital fluorescent stain. Cells incubated with mimosine for 1 hour showed only 5% protection, which increased to 13-17% between 3-21 hours incubation.

In the cultured HEK model, inhibition of DNA-PK by a chemical protein kinase inhibitor, dimethylaminopurine, accelerates SM (300 μM, 16-20 hours)-induced caspase-3 processing as well as PARP cleavage, the two biochemical hallmarks of apoptosis. This effect of DNA-PK inhibition on SM-induced apoptosis was confirmed using a cell clone lacking DNA-PK (Ray *et al*., 2002). We examined the effect of apoptosis inhibition on DNA damage/repair in SM (1 mM)-exposed HEK. As mentioned earlier, we have shown that the DNA repair enzyme DNA ligase I is activated via DNA-PK-mediated phosphorylation in response to DNA damage due to SM. We previously showed that in HEK, DNA ligase I activation following SM exposure is a time-dependent response, being maximum at 30 minutes to 1 hour after exposure and then decaying with time to almost the basal level in 4 hours when significant DNA repair was observed (Bhat *et al*., 1997). We, therefore, utilized the extent of phosphorylation as an indicator of enzyme activation and hence DNA damage. We measured DNA ligase I phosphorylation in unexposed or SM-exposed cells by affinity chromatography using a monoclonal bovine DNA ligase I antibody and ^{33}P-labeled DNA ligase I in cell-free extracts obtained from HEK exposed to SM in the presence of ^{33}P-labeled inorganic phosphate. We observed that SM (1 mM) increases enzyme phosphorylation that is prevented by treatment of cells with the general caspase inhibitor Z-VAD-FMK (Benzyl oxycarbonyl-Val-Ala-Asp (o-methyl-fluoromethylketone)). The Fas (CD95) antibody that blocks the Fas-mediated pathway of apoptosis also prevented SM-induced DNA ligase I phosphorylation. These results suggest that in SM-exposed cells, inhibiting apoptosis decreases DNA damage, indicating a signaling mechanism between DNA damage and apoptosis involving caspases.

We have discussed that SM vesication is accompanied by a separation of the basal keratinocytes from the basement membrane. Ray *et al*. (2003) and others (Smith *et al*., 1997) have proposed that the detachment of basal keratinocytes from the basement membrane in SM-

exposed skin could be the result of proteolytic degradation of the attachment proteins, particularly laminin-5. Some researchers have suggested that this state of "Anoikis," or homelessness, may trigger apoptosis of the detached cells (Frisch and Francis, 1994). Ray *et al*. (2003) have demonstrated that SM-stimulated protease degrades laminin-5 and a caspase inhibitor (Z-VAD-FMK) decreases laminin-5 degradation triggered by SM. These results suggest a possible relationship between SM-stimulated protease, laminin-5 degradation, and apoptosis. However, it is intriguing whether SM itself induces basal cell apoptosis or epidermal-dermal separation triggers apoptosis. These questions can be answered only by a thorough investigation of the sequence and the time-dependence of the effects involved.

In our studies *in vitro* in the HEK model, we have seen valid evidence of SM-induced apoptosis. We have gold-standard electron microscopic morphological evidence of apoptosis due to SM exposure (300 μM, 8 hr) to include both ultrastructural changes, e.g., nuclear and chromatin condensation, and morphological changes, e.g., formation of apoptotic bodies. We have established a rapid *in vitro* apoptosis assay using monolayer cell culture and fluorometric active caspase-3 determination. Using this assay, we have demonstrated SM-induced caspase-3 activation in a SM concentration- and time-dependent manner; this caspase-3 activation is blocked by the relatively specific peptide caspase-3 inhibitor AC-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-CHO(aldehyde)), the general caspase inhibitor Z-VAD-FMK, and the Ca^{2+} cheletor BAPTA-AM (1,2 - bis (o - aminophenoxy) ethane - N, N, N', N' - tetraacetic acid). As a functional correlate of caspase-3 activation, we have shown that in cultured HEK, SM (300 μM, 18 hours) causes LDH release, an indicator of cytotoxicity and cell viability loss that is partially prevented by both caspase-3 inhibitor AC-DEVD-CHO and the general caspase inhibitor Z-VAD-FMK. These *in vitro* experimental capabilities should allow us to validate our concepts and obtain useful information including prospective drug selection for *in vivo* validation in an appropriate animal model.

To offer a valid justification in support of the concept that an antiapoptotic antivesicant approach is worth considering we finally refer to a recently published report (Rosenthal *et al*., 2003). In this report, we presented conclusive evidence to demonstrate in the *in vivo* mouse skin model that SM causes basal cell apoptosis accompanied by caspase-3 activation, events that are clearly seen in the blister zone. Both SM-induced basal cell apoptosis and blistering are prevented in skin grafted onto nude mice utilizing human keratinocytes stably transfected with a dominant-negative Fas (death receptor)-associated death domain (FADD-DN) construct to block the Fas receptor pathway of apoptosis due to SM. Moreover, neither apoptosis nor vesication occurs in SM-exposed Fas knockout mouse skin that necessarily lacks the Fas pathway of apoptosis.

CONCLUSION

We have discussed that the vesicant SM is a high priority chemical warfare/terrorism threat. At present, there is no adequate vesicant medical pretreatment or therapy. The results from both *in vitro* and *in vivo* experimental models suggest a role of apoptosis in SM vesication and

tangible benefits of antiapoptotic intervention. Therefore, the antiapoptotic vesicant intervention strategy appears to be a worthwhile concept.

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